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Role of autophagy in chemoresistance: Regulation of the ATM-mediated DNA-damage signaling pathway through activation of DNA–PKcs and PARP-1

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ABSTRACT

Capsaicin treatment was previously reported to reduce the sensitivity of breast cancer cells, but not normal MCF10A cells, to apoptosis. The present study shows that autophagy is involved in cellular resistance to genotoxic stress, through DNA repair. Capsaicin treatment of MCF-7 cells induced S-phase arrest and autophagy through the AMPKα–mTOR signaling pathway and the accumulation of p53 in the nucleus and cytosol, including a change in mitochondrial membrane potential. Capsaicin treatment also activated δ-H2AX, ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase catalytic subunit (DNA–PKcs), and poly(ADP-ribose) polymerase (PARP)-1. Genetic or pharmacological disruption of autophagy attenuated capsaicin-induced phospho-ATM and phospho-DNA–PKcs and enhanced apoptotic cell death. ATM inhibitors, including Ku55933 and caffeine, and the genetic or pharmacological inhibition of p53 prevented capsaicin-induced DNA–PKcs phosphorylation and stimulated PARP-1 cleavage, but had no effect on microtubule-associated protein light chain 3 (LC3)-II levels. Ly294002, a DNA–PKcs inhibitor, boosted the capsaicin-induced cleavage of PARP-1. In M059K cells, but not M059J cells, capsaicin induced ATM and DNA–PKcs phosphorylation, p53 accumulation, and the stimulation of LC3II production, all of which were attenuated by knockdown of the autophagy-related gene *atg5*. Ku55933 attenuated capsaicin-induced phospho-DNA–PKcs, but not LC3II, in M059K cells. In human breast tumors, but not in normal tissues, AMPKα, ATM, DNA–PKcs, and PARP-1 were activated and LC3II was induced. The induction of autophagy by genotoxic stress likely contributes to the sustained survival of breast cancer cells through DNA repair regulated by ATM–mediated activation of DNA–PKcs and PARP-1.

1. Introduction

Capsaicin, the main compound in the capsaicinoids of red chili peppers, has been shown to be a chemopreventive agent both in vitro [1–4] and in vivo [4–6]. The molecular mechanisms for its anticancer effects were reviewed extensively by Oyagbemi et al. [7]. However, capsaicin also has carcinogenic, co-carcinogenic, and tumorigenic properties, as demonstrated by in vivo and epidemiological studies [8–10]. These apparently contradictory effects of capsaicin remain to be explained.

Autophagy, a lysosome-dependent degradation process, supplies energy to cells and plays a key role in cellular survival in response to various forms of stress [11]. It has thus been considered as a novel target in cancer treatment [12]. In general, the success of cancer treatments such as chemotherapy and radiotherapy is contingent on preventing tumor cell resistance, which is mediated by the exploitation of cellular survival mechanisms, including the downregulation of pro-apoptotic genes, overexpression of pro-survival genes, and induction of DNA repair pathways. Several studies have demonstrated the involvement of autophagy in DNA damage. The inhibition of DNA-dependent protein kinase catalytic subunit (DNA–PKcs), a key DNA-repair protein, was shown to sensitize human malignant glioma cells to radiation-induced autophagy [13]. An autophagy inhibitor, bafilomycin A1, sensitized U373-MG glioma cells to the alkylating agent telozolomide [14]. DNA mismatch repair induces autophagy in response to a genotoxic methylating agent, 6-thioguanine, and thus enhances the survival of human colorectal and endometrial cancer cells [15]. Camptothecin, a nuclear

Abbreviations: ATM, ataxia telangiectasia mutated; DNA–PKcs, DNA-dependent protein kinase catalytic subunit; PARP-1, poly(ADP-ribose) polymerase; PAR, anti-poly(ADP-ribose); 3-MA, 3-methyladenine; 3-AB, 3-aminobenzamide; AMPKα, AMP-activated protein kinase-α; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA; MTT, 3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide.

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topoisomerase 1 inhibitor, induced autophagy which causes delay apoptosis or prolong survival in MCF-7 cells [16]. Together, these studies suggest an important role for autophagy in DNA damage responses, but the underlying molecular mechanisms remain unclear.

In a previous study, we showed that the breast cancer cell lines MCF-7 and MDA-MB-231, when treated with capsaicin, were both less sensitive to apoptosis compared with non-transformed MCF10A cells and were proficient in autophagy induction [17], suggesting a role for autophagy in the protective signaling pathways that enable tumor cells to avoid apoptosis. To date, little is known about the role of autophagy in the molecular mechanisms that confer cellular resistance to chemotherapeutics.

In this study, we used human breast cancer cells to examine the role of autophagy in the DNA-damage signaling pathway after genotoxic stress and to investigate the underlying molecular mechanisms. Capsaicin-induced autophagy was found to regulate the DNA-repair signaling pathway via ataxia telangiectasia mutated (ATM)-mediated links to DNA–PKcs and PARP-1, thereby prolonging cancer cell survival.

2. Material and methods

2.1. Cell lines, chemicals, and antibodies

MCF-7 and MCF10A mammary epithelial cell lines were obtained from KCLB (Korean Cell Line Bank of Cancer Research Institute at Seoul National University College of Medicine). Cells were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum, 50 μg penicillin/ml, and 50 μg streptomycin/ml, at 37 °C in a 5% CO_2/95% air humidified incubator. 3-MA, 3-AB, MTT, caffeine, bafilomycin, E64d, pepstatin A, and pifithrin-a were purchased from Sigma–Aldrich (St. Louis, MO). Ku55933 and JC-1 were from Calbiochem (La Jolla, CA) and Molecular Probe (Eugene, OR), respectively. Antibodies against ATG8b/LC3B and PAR were supplied by Absent (San Diego, CA) and BD Pharmingen (San Diego, CA), respectively. Antibodies against γH2AX, phospho-AMPKα, p27, phospho-p53, p53, mTOR, and phospho-mTOR were from by Cell Signaling Technology (Danvers, MA). Anti-phospho-ATM and anti-ATM antibodies were purchased from Abcam (Cambridge, MA). Antibodies against DNA–PKcs, phospho-DNA–PKcs, AMPKα, p70S6K, phospho-p70S6K, and PARP-1 were obtained from Epitamics (Burlingame, CA). Antibodies against β-actin, Atg5, CD1, p21, p62, β-tubulin, goat anti-mouse IgG and goat anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HDAC1 antibody was purchased from GeneTex. Capsaicin (Sigma–Aldrich) was prepared as a 400 μM stock solution in DMSO (Sigma–Aldrich) and diluted with medium to give final concentrations of 0.025–0.001% DMSO, which had no cytotoxic effects on the cells. Other chemicals used in this study were of the purest grade available from Sigma–Aldrich.

2.2. Cytotoxicity assay, flow cytometry, and subcellular fractionation

The MTT assay, cell cycle analysis, and nuclear, mitochondrial, and cytosol fractionation were performed as described previously [17,18].

2.3. Transfection

Transient transfection was performed as previously described [17]. Briefly, cells were transfected with siRNA targeted against human APG5L (5′-GGACGAAUUCCAACUUGUU-3′) and human p53 (5′-UUACACAUGUAGUUGUAGUGGAUGG-3′) or the universal control siRNA (Ambion, Austin, TX) using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA). The cells were used 24 h after transfection.

2.4. Electron microscopy

Ultrastructural analysis was performed as described previously [19].

2.5. Immunofluorescence staining

Cells cultured on coverslips were fixed in 4% formaldehyde for 10 min, blocked with 3% normal goat serum, incubated with primary antibody overnight at 4 °C, visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Sigma–Aldrich), and counterstained with Hoechst 33342 (Sigma–Aldrich). The cells were then observed by fluorescence microscopy (Olympus, TH4-200).

2.6. Immunohistochemistry

Formalin fixed, paraffin embedded sections were deparaffinized in xylene and rehydrated in an ethanol gradient. After microwave epitope retrieval in 10 mM sodium citrate buffer (pH 6) for 10 min, the sections were incubated with p53 antibody (1:50) for overnight at 4 °C. A negative control with no primary antibody was performed for each specimen. Endogenous peroxidase activity was abolished by incubating the sections for 15 min in 0.3% H_2O_2. Next procesures were performed using Polink-2 AP broad detection kit according to the supplier's protocol (Life Science Division, WA, USA). The slides were counterstained with hematoxylin.

2.7. Comet assay

Cells were trypsinized and resuspended in PBS. Twenty microliters of the cell suspension (1×10^5 cells) were mixed with 200 μl low-melting-point agarose (0.5% (w/v) in PBS) and embedded onto comet slide (Trevision, Gaithersburg, MD). The agarose-cell suspension had solidified at 4 °C for 10 min. The slides were then immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10) for 1 h at 4 °C in the dark and then placed in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA) for 30 min at 4 °C to denature DNA. Electrophoresis was carried out at 4 °C for 30 min at 300 mA. The slides were then washed with 70% ethanol for 5 min and stained ethidium bromide (20 μg/ml). Cells were analyzed at ×200 magnification using a fluorescence microscope equipped with a green filter.

2.8. Immunoblot analysis

Cell lysates were immunoblotted as described previously [19]. Tissue samples were dissected from frozen specimens, homogenized in lysis buffer with a Dounce homogenizer, clarified by centrifugation at 10,000 × *g* for 20 min, and quantified for protein content. Proteins were separated by SDS-PAGE in 6–15% acrylamide gels. Subsequent procedures were performed as described for cell lysates.

2.9. Case selection and review

Invasive ductal breast carcinoma tissues were provided by the Biobank of Chonbuk National University Hospital. All samples were obtained with informed consent under institutional review board (IRB)-approved protocols. Specimens handling was approved by the IRB ethics board of Chosun University (IRB# 10-005).

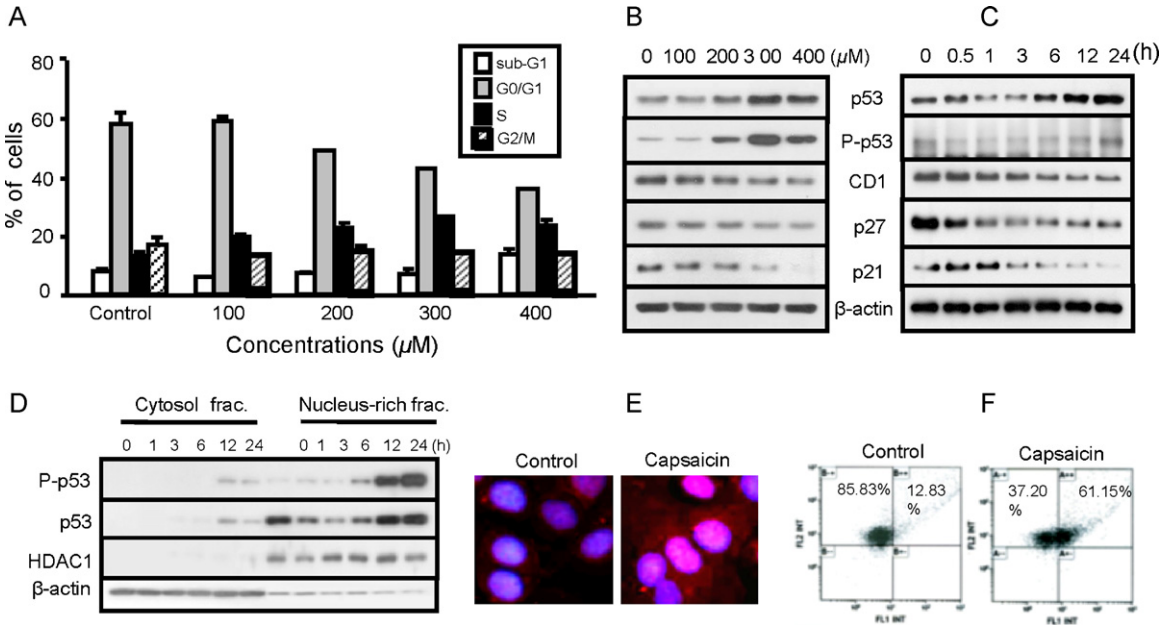

Fig. 1. Effect of capsaicin on S-phase arrest and autophagy induction in MCF-7 cells. (A) Flow cytometric analysis of PI-stained nuclei of cells treated for 24 h with the indicated concentrations of capsaicin. (B and C) Cells were treated with increasing concentrations of capsaicin for 24 h (B) or with 250 μM capsaicin (C), harvested, and analyzed by immunoblotting for the indicated proteins. (D) Capsaicin-treated (250 μM) cells were analyzed by immunoblotting for p53 and phospho-p53 expression in nucleus and cytosol fractions. (E) Cells cultured on coverslips were treated with capsaicin for 21 h and stained for p53 as described in Section 2. (F) Cells treated with 250 μM capsaicin for 21 h were harvested, stained with JC-1 (5 μg/ml), and analyzed by flow cytometry. Three separate experiments showed similar results. β-Actin and HDAC1 were used as loading controls for the cytosol and nucleus, respectively.

2.10. Statistical analysis

All experiments were repeated at least three times. Significant differences between treatments and the respective controls were determined based on Student's *t*-test. The values are expressed as means ± SD.

3. Results

3.1. Effects of capsaicin on the cell cycle and p53

To examine the cytotoxic effect of capsaicin, cells were treated with various concentrations of capsaicin and their cell cycle profiles were analyzed. Capsaicin caused S-phase arrest, corresponding to a decreased percentage of G0/G1 cells. There was a small increase in the number of sub-G1 cells among cells treated with 400 μM capsaicin (Fig. 1A). The expression levels of proteins regulating cell cycle progression were then examined. Dose-dependent accumulation of p53, as Ser15-phospho-p53, was observed, whereas CD1, p21, and p27 levels decreased (Fig. 1B). A kinetic analysis of the p53 level showed a biphasic pattern, with a gradual decrease after 3 h of capsaicin treatment followed by accumulation of the protein. By contrast, p21, p27, and CD1 decreased continuously (Fig. 1C). To further characterize capsaicin-induced p53 accumulation, the protein was analyzed in cytosol and nuclei-rich fractions. The increase in the p53 level was similar between the nuclei-rich fraction and the total lysate, while p53 accumulated later in the cytosol, but to a lesser extent (Fig. 1D). In cells treated with capsaicin for 21 h, p53 immunocytochemistry showed an increase in nuclear and cytoplasmic staining compared with control cell staining, and a reduction in mitochondrial membrane potential as demonstrated by JC-1 staining and fluorescence-activated cell sorting (Fig. 1E and F). Therefore, capsaicin appears to induce growth inhibition through S-phase arrest, and the subcellular localization of p53 suggests it has a dual role.

3.2. Capsaicin induces autophagy through the AMPKα–mTOR signaling pathway

Electron microscopy of capsaicin-treated cells showed vacuoles of various sizes containing cellular organelles; these might have been autophagosomes or autolysosomes (Fig. 2A). To confirm LC3 conversion by capsaicin, cells were treated with bafilomycin A1 (BaF1), an inhibitor of autophagosome-lysosome fusion, and E64d/pepstatin, lysosomal inhibitors, for 1 h prior to addition of capsaicin resulted in greater accumulation of LC3II (Fig. 2B). The autophagy induction was further confirmed by Atg5 induction, LC3 conversion, and decreased p62 in a dose-dependent manner (Fig. 2C). Given that capsaicin induced Akt phosphorylation in MCF-7 cells [17], we examined AMP-dependent protein kinase α (AMPKα) and mammalian target of rapamycin (mTOR). AMPKα phosphorylation, mTOR dephosphorylation, and ultimately downregulation of phospho-p70S6K were observed (Fig. 2C). By contrast, MCF10A cells did not show altered mTOR or AMPKα phosphorylation, or the induction of phospho-p70S6K (Suppl. Fig. 1).

To examine the role of capsaicin-induced autophagy, MCF-7 cells were pretreated with the autophagy inhibitor 3-MA for 2 h and continuously treated with capsaicin for 24 or 48 h and then stained with propidium iodide (PI) and Hoechst 33342. Apoptotic cells accounted for 0.45% of untreated cells, but the proportion increased to 5.09% and 11.6% in cells treated with capsaicin for 24 and 48 h, respectively. The level of apoptosis in cells treated with capsaicin plus 3-MA increased to 10.4% and 21.7% at 24 and 48 h, respectively (Fig. 2D). These results suggest that capsaicin induces autophagy through the AMPKα–mTOR signaling pathway and thereby alters cell survival by blocking apoptosis.

3.3. Capsaicin induces PARP-1 activation and inactivation

DNA strand breaks activate PARP-1, which is involved in DNA repair or cell death depending on the extent of DNA damage [20]. As shown in Fig. 3A, PARP-1 levels increased after 30 min of capsaicin

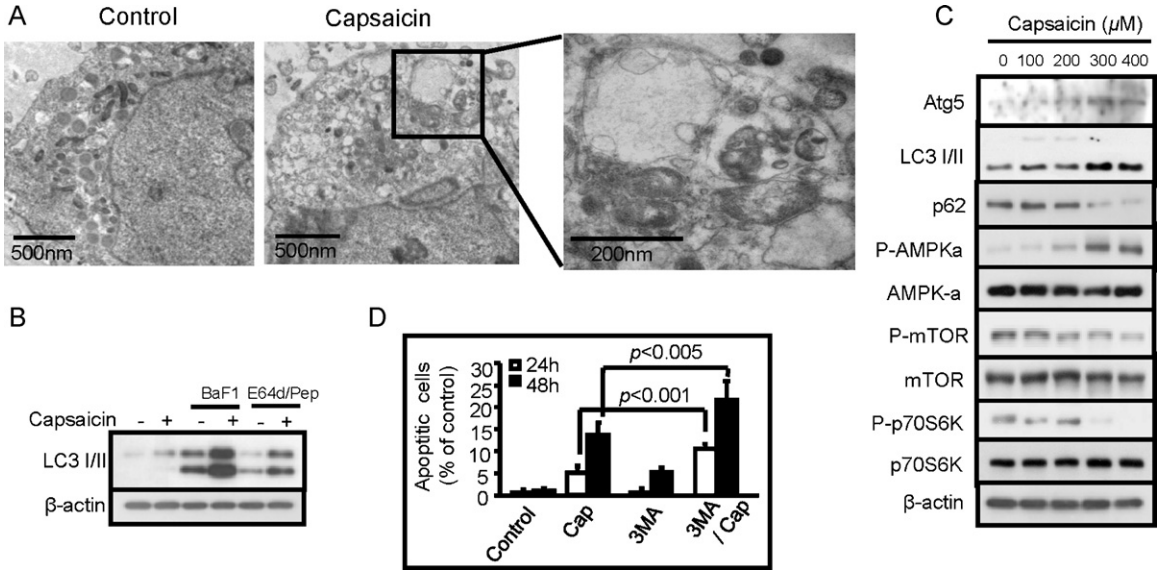

Fig. 2. Capsaicin-induced autophagy is induced by the AMPKα–mTOR signaling pathway, which is involved in cell survival. (A) Ultrastructural features of capsaicin-treated MCF-7 cells. Cells treated with 250 μM capsaicin for 21 h were fixed and processed for electron microscopy. (B) Cells were treated with 250 μM capsaicin for 8 h after pretreatment with BaF1 (50 nM) and E64d (10 μg/ml)/pepstatin A (10 μg/ml) for 1 h. LC3 II markedly accumulated in the presence of BaF1 and E64d/pepstatin A. (C) Cells were treated with increasing concentrations of capsaicin for 24 h, and cell lysates were immunoblotted for the indicated proteins. (D) Autophagy disruption enhanced apoptotic cell death. Cells treated with 10 mM 3-MA for 2 h and 250 μM capsaicin for 24 or 48 h were stained with PI and Hoechst 33342. Three random fields per condition were examined by fluorescence microscopy and photographed. Data are reported as means ± SD from three independent experiments.

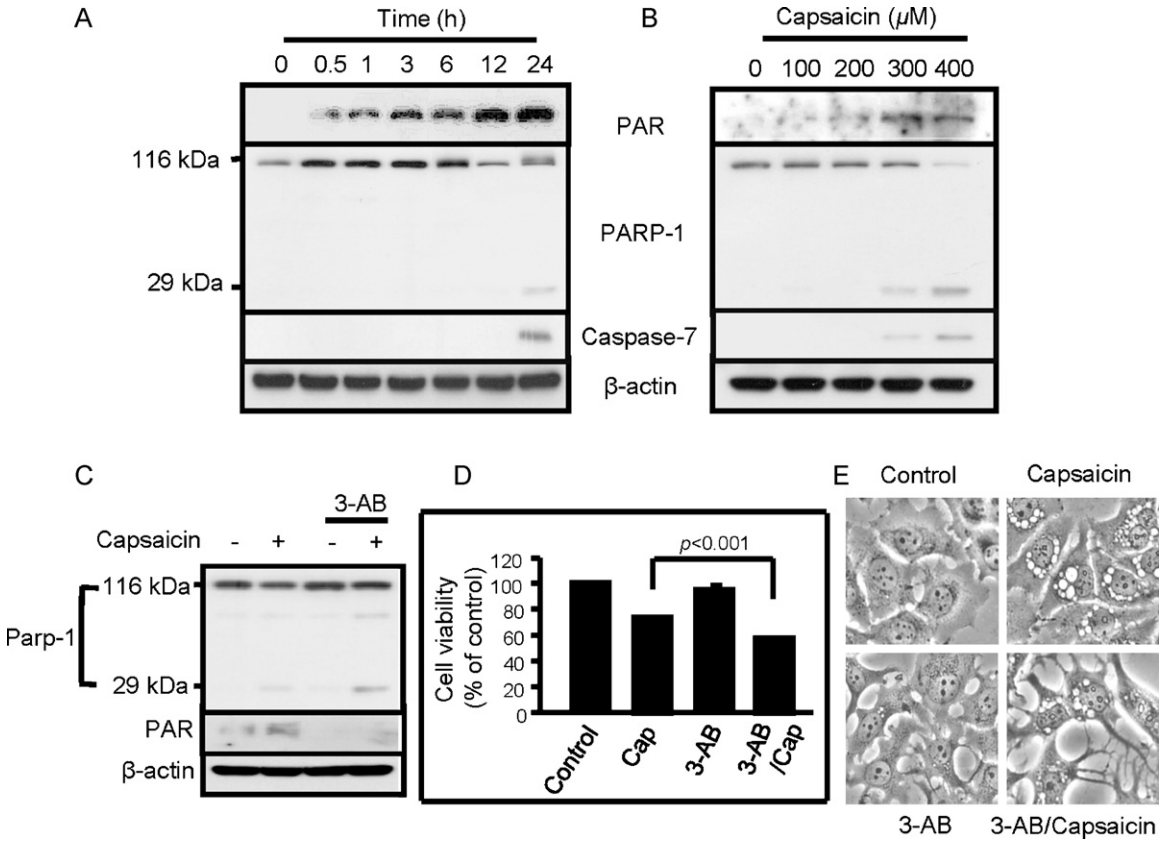

Fig. 3. Capsaicin induces PAR formation and PARP-1 cleavage in MCF-7 cells. (A and B) Cells were treated as described in Fig. 1B and C. Cell lysates were immunoblotted for PARP-1, and the membranes were stripped and reprobed for PAR. (C) Blocking of PAR formation enhanced PARP-1 cleavage. Cells were pretreated with 10 mM 3-AB for 2 h before the addition of capsaicin for 21 h. The cells were harvested and immunoblotted for PARP-1 and PAR. (D) Cells seeded in a 48-well plate were cultured for 24 h, treated as described in (C), and then analyzed by MTT assay. Data are reported as means ± SD of the average percentage increase vs. the untreated control, from three independent experiments. (E) Pretreatment with 10 mM 3-AB did not block capsaicin-induced cytoplasmic vacuolization, but induced severe cell shrinkage (×200).

treatment and decreased abruptly at 12 h. However, the 29-kDa PARP-1, which is the active form resulting from caspase-7 cleavage [21], was not detected until 24 h later. Therefore, the involvement of PARP-1 in DNA repair was examined. In membranes stripped of PARP-1 and reprobed with anti-poly(ADP-ribose) (PAR) antibody, PAR increased with time, indicating that PARylated PARP-1 was not detected. Activation and cleavage of PARP-1 were confirmed in a dose-dependency experiment (Fig. 3B). The decrease in 116-kDa PARP-1 by PARylation was confirmed using the poly(ADP-ribosyl)ation inhibitor 3-AB, which completely blocked PAR formation. In addition, compared with capsaicin-treated cells, the 3-AB-treated cells showed slightly increased levels of 116-kDa PARP-1 with increasing levels of the 29-kDa form, and cell death was ultimately enhanced (Fig. 3C and D). However, 3-AB did not inhibit capsaicin-induced cytoplasmic vacuolization (Fig. 3E). These results suggest that capsaicin-induced PARP-1 activation is involved in cell protection.

3.4. Capsaicin induces phosphorylation of γH2AX, ATM, and DNA–PKcs

Capsaicin-treated MCF-7 cells were less sensitive to apoptosis and activated PARP-1, suggesting that capsaicin induces the DNA-repair signal. Immunostaining for γH2AX, a marker of DNA double-stranded breaks (DSBs), in capsaicin-treated cells showed nuclear staining (Fig. 4A). Alkaline comet assay showed an increased tail migration in capsaicin-treated cells (Fig. 4B). H2AX phosphorylation or p53 accumulation requires ATM activation [22,23], and Ser1981-phospho-ATM was present in capsaicin-treated cells (Fig. 4C), as was Tyr2609-phospho-DNA–PKcs, a marker of DNA DSBs [24]. These results were confirmed in a dose-dependency experiment (Fig. 4D). In MCF10A cells treated with 300 or 400 μM capsaicin, phospho-ATM increased only slightly and phospho-DNA–PKcs was not upregulated (Suppl. Fig. 1). Therefore, capsaicin may induce the DNA-repair signaling pathway via the activation of ATM and DNA–PKcs.

3.5. Capsaicin-induced autophagy regulates p53, DNA–PKcs, and PARP-1 through ATM activation

To determine whether autophagy contributes to the capsaicin-induced DNA-damage signal, MCF-7 cells were pretreated with 3-MA for 2 h and then with capsaicin for 21 h. Morphological observations showed that capsaicin induced the formation of cytoplasmic vacuoles of various sizes and that this process was completely blocked by 3-MA treatment, which also caused cellular shrinkage. Co-treatment with 3-MA and capsaicin induced cellular shrinkage as well as the formation of round floating cells (Fig. 5A). Addition of 3-MA inhibited capsaicin-induced LC3 conversion and GFP-LC3 dots (Suppl. Fig. 2). Correspondingly, the level of p62 protein decreased with capsaicin treatment and increased with 3-MA addition. Furthermore, 3-MA attenuated the capsaicin-induced activation of p53, ATM, and DNA–PKcs, but increased 29-kDa PARP-1 (Fig. 5B). This was confirmed by the transfection of *atg5* siRNA (Fig. 5C, Suppl. Fig. 3), and *atg7* siRNA (Suppl. Fig. 4).

Next, to confirm whether ATM occurs downstream of autophagy, MCF-7 cells were treated with caffeine or Ku55933, which are both inhibitors of ATM kinase [25,26]. Neither inhibitor blocked capsaicin-induced cytoplasmic vacuolization and GFP-LC3 dots but each caused cellular shrinkage and the formation of round

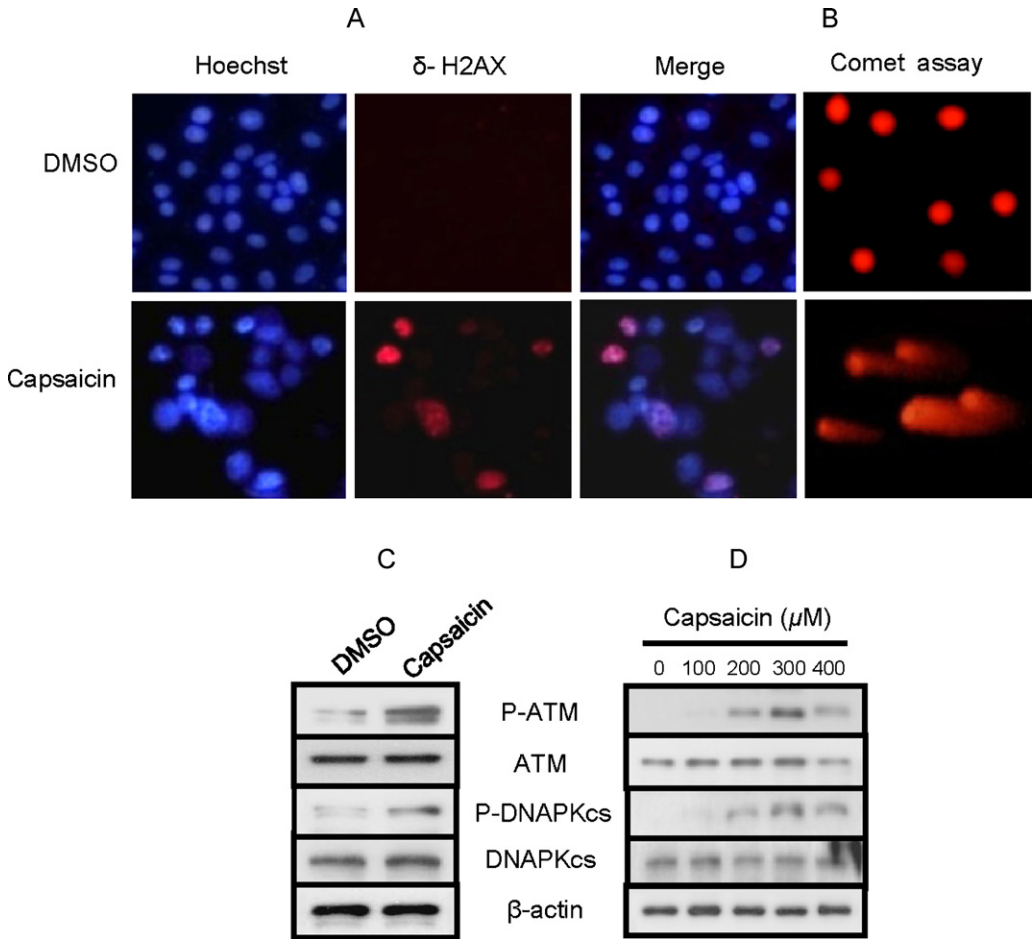

Fig. 4. DNA breakage and DNA repair markers induced by capsaicin. (A) MCF-7 cells grown on coverslips were treated with 250 μM capsaicin for 6 h, fixed, and stained for γH2AX and with Hoechst 33342. (B) Cells were treated with 250 μM capsaicin for 12 h, trypsinized, and applied to comet assay as described in Section 2. (C and D) Cells were treated with 250 μM capsaicin for 21 h or with increasing concentrations of capsaicin for 24 h and then immunoblotted with the indicated antibodies.

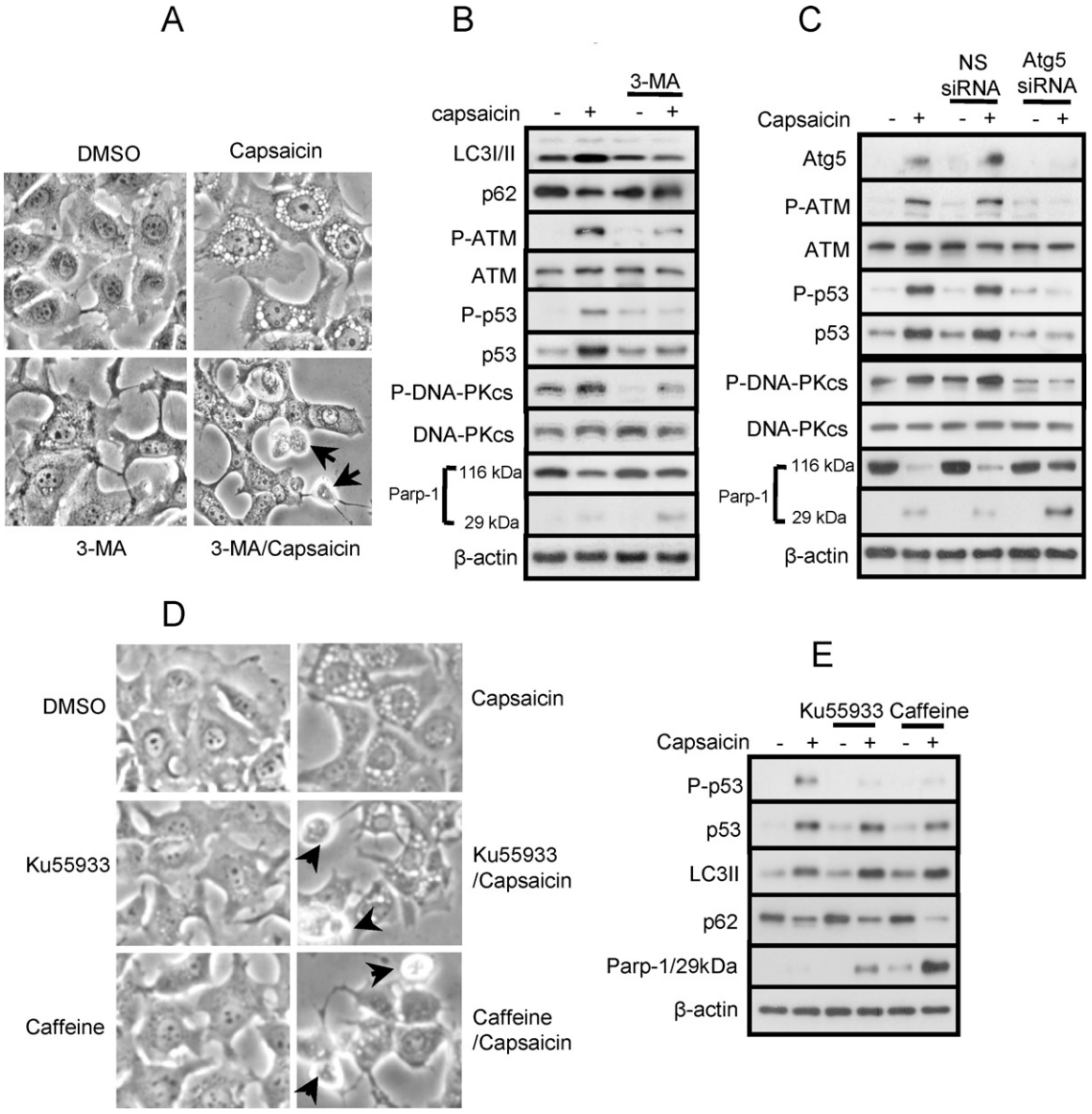

Fig. 5. ATM regulates p53, DNA–PKcs, and PARP-1 activation downstream of autophagy. (A) Massive cytoplasmic vacuolization in cells treated with 250 μM capsaicin for 21 h was completely inhibited by pretreatment with 10 mM 3-MA for 2 h (×200). (B and C) Cells pretreated with 3-MA or transfected with control siRNA or siRNA for Atg5 (10 pM) were treated with 250 μM capsaicin for 21 h and immunoblotted with the indicated antibodies. (D) Treatment with Ku55933 or caffeine did not block capsaicin-induced cytoplasmic vacuolization. (E) Cells pretreated with 30 μM Ku55933 or 2 mM caffeine for 1 h were further treated with 250 μM capsaicin for 21 h and then immunoblotted with the indicated antibodies.

floating cells (Fig. 5D, arrows, Suppl. Fig. 2). In addition, each attenuated capsaicin-induced phospho-p53, a marker of ATM activity [25], and increased 29-kDa PARP-1, but had no effect on LC3II and p62 (Fig. 5E). Therefore, capsaicin-induced cytoplasmic vacuoles may be autophagic, and autophagy appears to be involved in the capsaicin-induced DNA-damage signaling pathway, through ATM-mediated activation of p53, DNA–PKcs, and PARP-1.

3.6. Capsaicin-induced p53 regulates PARP-1 and DNA–PKcs activation

According to the above results, capsaicin-induced autophagy regulates p53, DNA–PKcs, and PARP-1. To determine the order of regulation, p53 was blocked using pifithrine (PFT)-α. This treatment attenuated capsaicin-induced phospho-53 and phospho-DNA–PKcs, and increased PARP-1 cleavage, but it had no effect on LC3II and p62 (Fig. 6A, Suppl. Fig. 2). These results were confirmed in cells transfected with p53 siRNA (Fig. 6B). Microscopy of the PFT-α-treated (Fig. 6C) or p53 siRNA-transfected cells (Suppl. Fig. 5) showed no inhibition of capsaicin-induced cytoplasmic vacuolization. PFT-α alone did not affect cell growth compared with vehicle-treated cells. By contrast, co-treatment with PFT-α and capsaicin decreased cell growth significantly compared with capsaicin-treated cells, in which apoptosis increased (Fig. 6D and E). Treatment with Ly294002, a specific inhibitor of DNA–PKcs [27], had no effect on p53, but enhanced PARP-1 cleavage and ultimately increased apoptosis (Fig. 6F and G). This result indicates that capsaicin-induced p53 regulates the activation of DNA–PKcs and PARP-1, which are involved in cell protection.

3.7. Capsaicin induces autophagy and the ATM–DNA–PKcs signaling pathway in human glioma M059K cells.

The linkage of ATM to the DNA–PKcs signaling pathway in capsaicin-induced cell protection was confirmed in human malignant glioma M059K cells, which express DNA–PKcs, and in

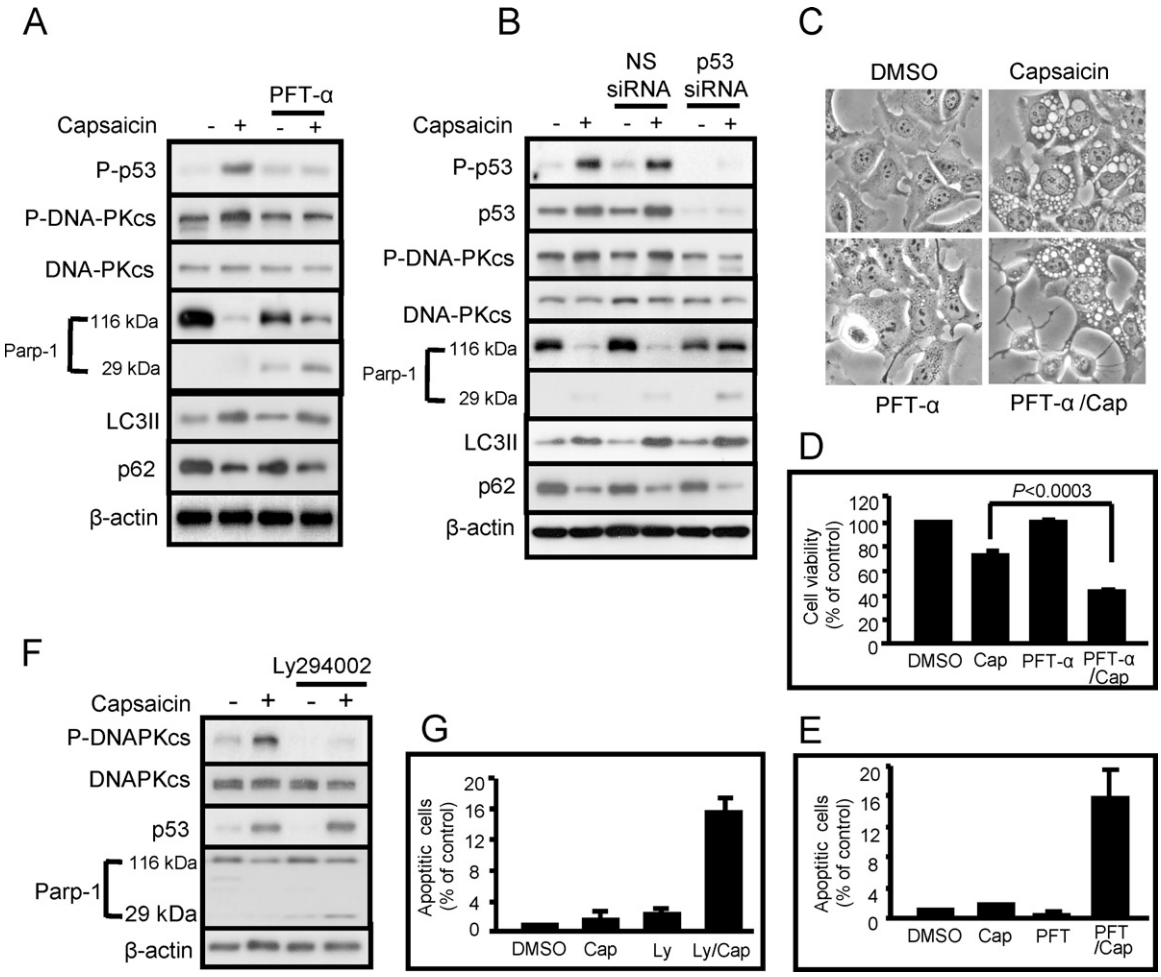

Fig. 6. Capsaicin-induced p53 regulates DNA–PKcs and PARP-1. (A) Cells treated with 20 μM PFT-α for 1 h (A) or transfected with control siRNA or p53 siRNA (10 pM) (B) were treated with 250 μM capsaicin for 21 h and then immunoblotted for the indicated proteins. (C) Pretreatment with PFT-α did not inhibit capsaicin-induced cytoplasmic vacuolization. (D and E) Cells were treated as described in (A) and then either tested for cell viability by MTT assay or stained with PI and Hoechst 33342. (F) Cells pretreated with 50 nM Ly294002 for 1 h were treated with 250 μM capsaicin for 21 h and then immunoblotted for the indicated proteins. (G) Cells seeded in a 48-well plate were cultured for 21 h, treated as described in (F), and stained with PI and Hoechst 33342. The number of cells with a fragmented nucleus was determined. Data are means ± SD of the average percentage increase over the untreated control in three independent experiments.

DNA–PKcs-deficient M059J cells. Capsaicin-treated M059K cells showed phosphorylation of DNA–PKcs, ATM, and p53, and increased LC3II, in a dose-dependent manner. In M059J cells treated with 300 μM capsaicin, the LC3II was induced, but the cells were sensitive to apoptosis, as demonstrated by FACS analysis (Fig. 7A and B). Knockdown of *atg5* in M059K cells attenuated capsaicin-induced LC3II and increased p62 compared with control siRNA-transfected cells, and attenuated capsaicin-induced phosphorylation of ATM, DNA–PKcs, and p53 (Fig. 7C). Furthermore, Ku55933 treatment inhibited phosphorylation of ATM, p53, and DNA–PKcs, but did not affect LC3II and p62 (Fig. 7D). These findings suggest that the role of autophagy in capsaicin-induced cell protection depends on the ATM–DNA–PKcs signaling pathway.

3.8. Induction of autophagy and DNA repair markers in human breast cancer tissues

To determine whether autophagy contributes to breast cancer, invasive ductal carcinoma tissues and normal tissues adjacent to breast carcinomas were obtained from biopsies of 10 women with breast cancer. Various intensities of immunoreactivity of phosphorylated DNA–PKcs and ATM were seen only in the cancer tissues, which also exhibited downregulated PARP-1 in parallel with PAR formation. Increased LC3II was related to AMPKα activation and 70S6K dephosphorylation, unlike in normal tissues. However, antibodies against p53 (clone Do-1 or Do-7), which detect both wild-type and mutant protein, produced strong bands in all normal tissues, with Ser15-phospho-p53 and weak bands seen in cancer tissues (Fig. 8A). To confirm the Western blot analysis for p53, we tried to immunohistochemistry for p53 in human breast tissues. In normal tissue adjacent to carcinomas, strong immunoreactivity for p53 showed in the ductal epithelial cells (Fig. 8B, a). In the tumor tissue, p53 showed diffuse and weak staining pattern in the malignant ductal epithelial cells (Fig. 8B, b). In two cases, intense nuclear staining was seen in malignant epithelium. Ductal epithelial cells of non-cancerous tissues derived from fibrocystic breast condition showed weak (Fig. 8B, c, arrows) or negative staining (Fig. 8B, d black stars) pattern for p53. These results suggest that ATM-dependent p53 is associated with autophagy. β-actin and α-tubulin expressed highly in the cancer tissues and normal tissues, respectively.

4. Discussion

The present study shows that the DNA-repair signaling pathway is involved in cellular resistance to chemotherapeutic agents (Fig. 9). In mammals, mTOR is a gatekeeper of autophagy, sensing the level of nutrients or energy, and is regulated by Akt or AMPK [28,29]. In capsaicin-treated MCF-7, but not in MCF10A cells,

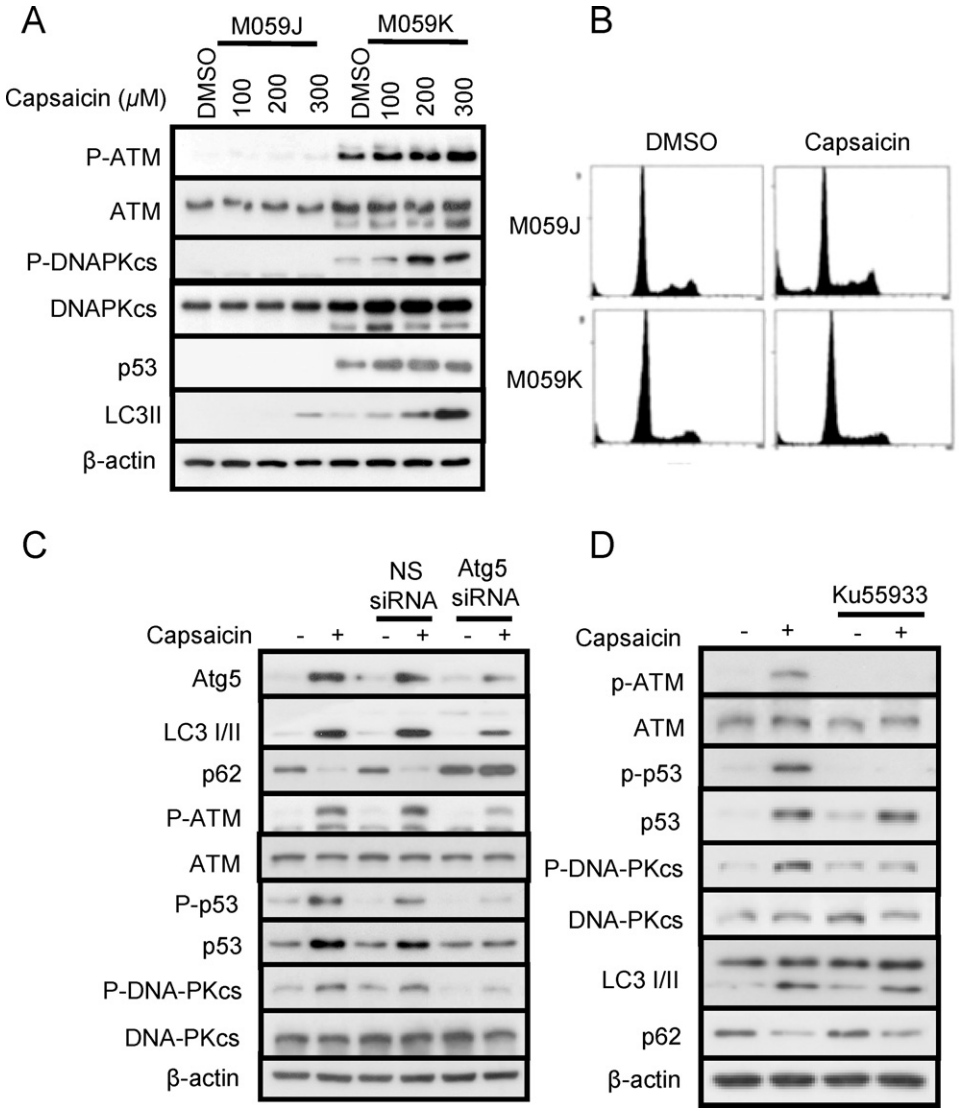

Fig. 7. Capsaicin-treated M059K cells activate ATM and DNA–PKcs and induce autophagy. (A and B) M059J and M059K cells were treated with increasing concentrations of capsaicin for 21 h and analyzed either by immunoblotting for the indicated proteins (A) or by flow cytometry to determine the sub-G1 population (B). (C) Cells transfected with Atg5 siRNA (10 pM) were treated with 200 μM capsaicin and analyzed for the indicated proteins. (D) Cells pretreated with 10 μM Ku55933 for 1 h were exposed to 200 μM capsaicin for 21 h and then analyzed by immunoblotting for the indicated proteins.

AMPKα was phosphorylated, mTOR was dephosphorylated, and autophagy was induced, indicating that capsaicin-induced autophagy is regulated by energy sensors. In addition, the disruption of autophagy enhanced apoptosis, implying that autophagy in malignant breast cells is involved in cell protection.

DNA-damage-induced cell cycle arrest promotes cell survival by enabling the repair of damaged DNA before cell cycle progression, and this mechanism may explain the resistance of tumor cells to chemotherapeutics [14,26]. On the other hand, irreparable DNA damage induces apoptosis [30]. In previous studies, capsaicin-induced G0/G1 arrest was associated with p53 phosphorylation and apoptosis in human esophageal epidermoid carcinoma cells, human urothelial cancer cells, and human leukemic cells [31–33]. Consistently, capsaicin-treated MCF-7 cells accumulated Ser15-phospho-p53, whereas neither Bax nor Bid was activated; instead, BCl2 levels were increased (data not shown). Under normal conditions, p53 is regulated by MDM2-mediated proteasomal degradation [34]. In MCF-7 cells, early capsaicin-induced p53 degradation was blocked by a distinctive signal, with p53 levels showing a biphasic pattern. The accumulation of p53 was not attributable to proteasomal dysfunction; the dose- and time-dependent degradation of p21, a proteasome substrate [35], in response to capsaicin established that proteasomal activity was still functional.

Although p53 is known to regulate autophagy [36–38], capsaicin-induced p53, while still regulating autophagy, was localized in the nucleus and, to a lesser extent, the cytosol of MCF-7 cells. This suggests a dual role of p53 in autophagy. DNA is most vulnerable to damage during S phase, when the chromosomes replicate, and p53 appears to be involved in S-phase arrest, thereby promoting DNA repair [39]. Capsaicin-induced S-phase arrest was decreased by 3-MA and PFT-α (Suppl. Fig. 6), and both inhibitors blocked capsaicin-induced p53 accumulation. Although our data do not clearly define the role of cytosolic p53, it seems that wild-type p53 can relocate to the mitochondria and trigger an apoptotic signaling pathway [40]. Indeed, changes in the mitochondrial permeability transition and the release of cytochrome C were followed by cytosolic p53 accumulation (data not shown), suggesting that transcriptional and non-transcriptional functions of p53. Therefore, apoptosis induction during autophagy may be involved in removing cells with irreparable DNA damage.

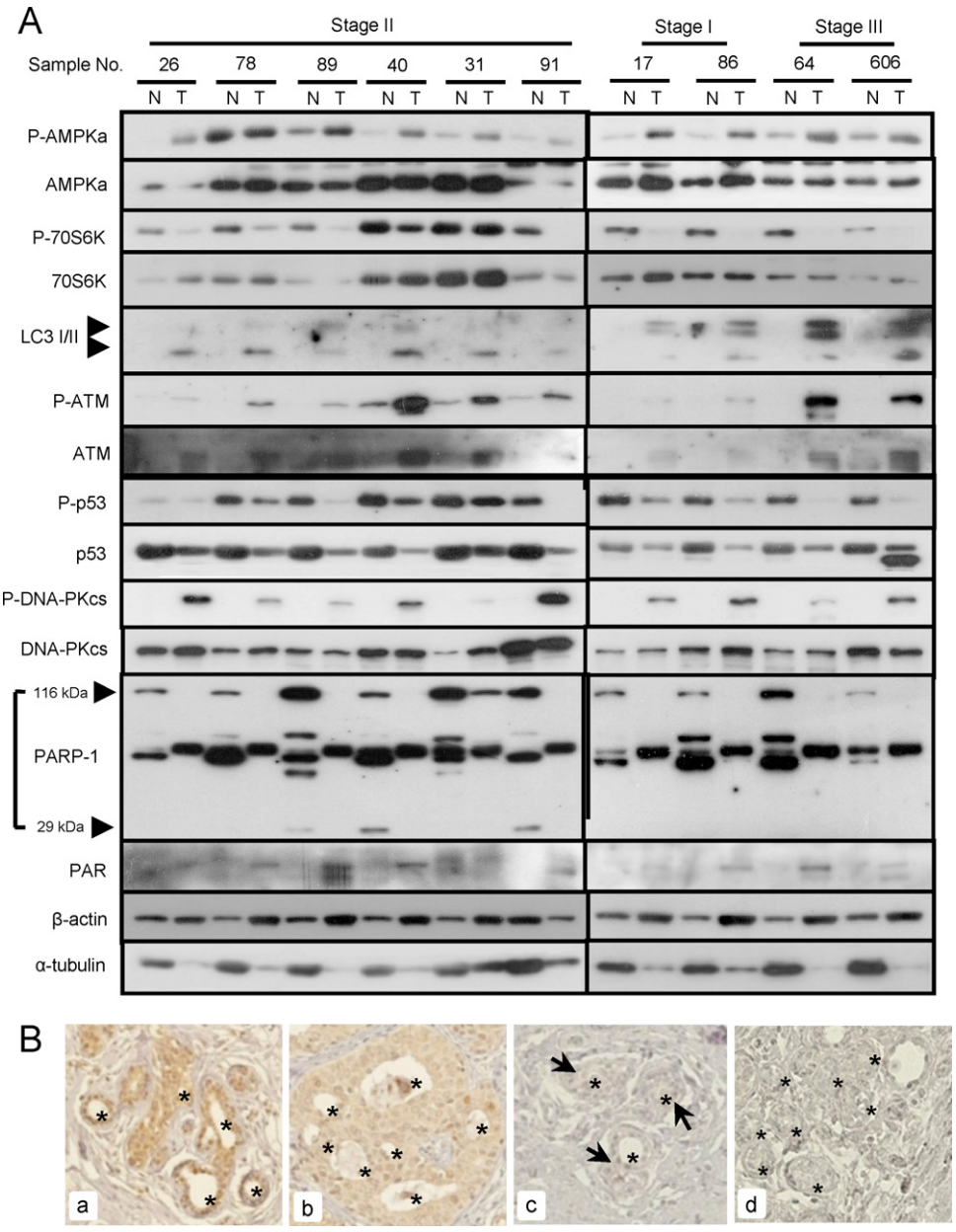

Fig. 8. Autophagy induction and expression of DNA damage markers in human breast cancer tissues. Tissue samples were processed as described in Section 2 and then analyzed by immunoblotting for the indicated proteins. β-Tubulin served as a loading control. The immunoblot data are representative of at least three independent experiments. N, normal tissues; T, tumor tissues. (B) Immunohistochemical study for p53 expression. Strong immunoreactivity for p53 showed in the ductal epithelial cells of normal breast tissue adjacent to carcinomas (a), and diffuse and weak staining pattern in the malignant ductal epithelial cells (b). Fibrocystic breast tissues showed weak (c, arrows) or negative staining (d, black stars) pattern for p53. Stars indicate mammary ducts.

PARP-1 activation following DNA damage causes the PARylation of several proteins, including PARP-1. PARP-1 participates in many molecular and cellular processes such as DNA damage detection, DNA repair, and carcinogenesis [41]. In addition, PARP-1 is involved in autophagy during DNA damage, thus promoting cell survival [42]. Capsaicin-induced downregulation of the full-length PARP-1 correlated well with PAR formation, and the PARP inhibitor 3-AB enhanced capsaicin-induced cell death. A role for the PARP inhibitors 3-AB and NU 1025 in cancer chemotherapy has been previously reported [43]. Here, autophagy disruption increased capsaicin-induced 25-kDa PARP-1, indicating that PARP-1 activation is regulated by autophagy. Also, the involvement of PARP-1 activation in cell survival was confirmed in human breast cancer tissue. PARylated PARP-1, accompanied by marked downregulation of PARP-1 and LC3II, was observed in all 10 cancer biopsies examined. Based on the data from MCF-7 cells, PARP-1 activation in human cancer tissues may be regulated by autophagy, but this remains to be confirmed in humans.

In mammalian cells, DNA–PK is composed of the catalytic subunit DNA–PKcs and the DNA-binding heterodimer Ku70/80, and plays important roles in the non-homologus end-joining pathway of DNA DSBs [26]. DNA–PKcs phosphorylation at Tyr2609 is needed for joining DSBs and for cell survival in response to irradiation (IR)-mediated DNA damage [44]. Therefore, capsaicin-induced DNA–PKcs appears to be associated with cell protection. Support for this was obtained in DNA–PKcs-expressing M059K cells. DNA–PKcs has also been reported to be a negative regulator of IR-induced autophagy in human malignant glioma cells [13]. On the other hand, our results showed that capsaicin-induced phospho-DNA–PKcs is attenuated by genetic or pharmacological

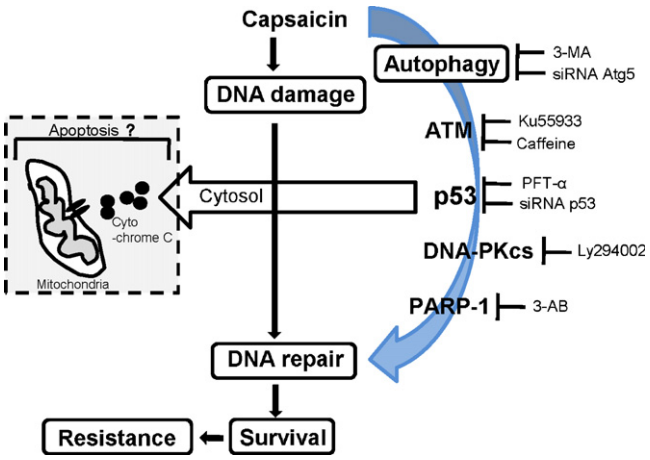

Fig. 9. A proposed schematic model on the DNA-damaging signaling pathway which involved in the cellular resistance to chemotherapeutic agents. Capsaicin-induced DNA damage triggers autophagy through AMPKα–mTOR signaling pathway which regulates activation of ATM, DNA–PKcs, and PARP-1, and consequently, cell survival. The possible signaling pathways induced by cytoplasmic p53 that were not confirmed in this work is indicated by dashed-line.

inhibition of autophagy. Furthermore, Ly294002 had no effect on capsaicin-induced LC3II, indicating that capsaicin-induced DNA–PKcs is regulated by autophagy. These findings contrast with those reported by Daido et al. [13] and may reflect differences in stimulation conditions.

ATM, a sensor of DNA damage, is required for the phosphorylation of p53 and DNA–PKcs [25,45], both of which are key regulators in the repair of IR-induced DNA DSBs. ATM kinase inhibition sensitized cells to IR or chemotherapeutics [28,46,47]. It was recently reported that cytoplasmic ATM regulates autophagy through mTOR, but the role of ATM-induced autophagy was not determined [48]. In the present study, ATM was regulated by autophagy in MCF-7 and M059K cells; ATM inhibitors had no effect on LC3II and increased PARP-1 cleavage, indicating that capsaicin-induced autophagy regulates ATM, which is involved in cell protection.

These findings suggest that DNA repair signaling is involved in the sustained survival of breast cancer cells, which was confirmed in human breast cancer tissues. In cancer tissues, but not in normal tissues, ATM, DNA–PKcs, and PARP-1 were activated and LC3II was induced. Ductal epithelial cells of normal tissues strongly expressed nuclear p53 and Ser15-phospho-p53, as demonstrated by immunohistochemistry and immunoblot analysis, respectively, but barely expressed ATM, suggesting that p53 levels in normal tissues are independent of ATM. Previous studies have suggested that p53 accumulation in non-malignant breast tissue is related to an increased risk for breast cancer [49]. Indeed, in tissue samples have fibrocystic change p53 was negative or very weak staining. A subcellular localization study of p53 in breast cancers showed that 30% of mutant p53 was localized in the nucleus, and approximately 70% was either non-detectable or appeared as diffuse nuclear and cytoplasmic staining [50]. In our immunohistochemistry study, 80% of human breast cancer tissues showed diffuse p53 staining, supporting the involvement of wild-type p53 in autophagy induction. As for an internal loading control, GAPDH and β-actin have been reported to express highly in the cancer tissues [51]. Consistently, we found high level of β-actin and GAPDH (not shown) in the cancer tissues of paired samples (cancer tissue and adjacent noncancer tissues), while α-tubulin and vimentin (not shown) were expressed highly in the normal tissues.

This is the first study to show that resistance to a chemopreventive agent, capsaicin, appears to be caused by DNA repair through autophagy-mediated ATM, p53, DNA–PKcs, and PARP-1 activation. The strong induction of DNA-repair signaling can interrupt the treatment of human breast cancer and thus may be an important consideration in therapy selection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.12.029.

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